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# Variation of transgene expression in plants

Peter Meyer
*Max-Delbrück-Laboratorium in der MPG, Carl-von-Linné Weg 10, D-50829 Köln, Germany*

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## Summary

Genetic engineering has proved to be a powerful technique for the identification of plant genes and the construction of new genetic traits of agricultural importance. A growing number of transgenic plants is available to the breeder for incorporation into breeding programmes and production of new material for the market. A prerequisite for marketing transgenic material, however, is the control of stable transgene expression. The analysis of mechanisms that influence transgene activity is therefore not only an interesting field in basic research, but also an important aspect for application.

This article summarizes work on the analysis of expression variegation in transgenic petunia plants that carry the maize A1 transgene. Although far from giving a final answer to the solution of the problem, the description of certain parameters involved in the regulation of transgene expression should provide some guidance for the prevention of expression variegation in transgenic plants.

## Introduction

One of the most critical aspects of molecular plant breeding is the long-term stable activity of recombinant genes that have been introduced into crop species. In classical breeding programmes the selection of a particular trait may be based on the activity of one or several genes often not identified at the molecular level. So far it is uncertain whether a lack of a particular trait is always necessarily due to a mutation of the corresponding gene, or if a structurally intact gene is no longer active. In molecular plant breeding, a recombinant molecule is transferred into the plant genome, encoding information for a protein that provides a defined structural or catalytic function. The transgene consists of a coding sequence of eukaryotic, prokaryotic or viral origin linked to a constitutive or developmentally-regulated promoter. By genetically engineering resistance genes, important agricultural traits have been designed and transferred, such as resistance against herbicides (Botterman & Leemans, 1988), insects (Fischhoff et al., 1987) or viral infection (Powell Abel et al., 1986). Based on increasing knowledge about the molecular organisation of plant genes (Schell, 1987) plant molecular biology also provided the tools to modify certain metabolic pathways and to create new traits (Mariani et al., 1990; Meyer et al., 1987; Gray et al., 1992).

The number of transgenic plants tested in field trials is increasing rapidly (Kareiva, 1993), but it can be anticipated that only a few primary transformants will enter the market. Most probably transgenic material will be used in breeding programmes to introduce the recombinant trait into a particular background or to combine different transgenes in one crop line. It is not enough to select a transformant that carries a recombinant transgene; the latter must function under various environmental conditions, in different genetic backgrounds and in successive generations.

The A1-gene marker has been employed to analyse certain parameters that influence transgene activity. Transfer of the A1-gene of *Zea mays* into the whitish flowered *Petunia hybrida* line R101, leads to salmon red flower pigmentation (Meyer et al., 1987). The A1 gene encodes dihydroflavonol reductase (DFR), an enzyme of the anthocyanin pathway. The receptor line R101 accumulates dihydrokaempferol, a substrate of the maize DFR. In transgenic plants expressing the

A1 gene, dihydrokaempferol is converted into leucopelargonidin, which is further processed by endogenous enzymes into a pelargonidin pigment. A1 activity can be followed by analysing the occurrence and stability of salmon-red pigmentation in the flower of transgenic plants. The system has the advantage that expression can be monitored in individual floral cells and that no staining procedure is required. Transgenic petunia plants can be propagated over several years via cuttings. Due to the continuous production of new flowers, expression of the transgene can be monitored over longer periods and under variable conditions.

The aim of this review is to summarize the data gained from expression studies based on the A1-system and to discuss certain approaches that target stabilization of transgene expression.

## Regulation of gene activity

Expression of a particular piece of genetic information requires a series of molecular mechanisms including transcription of a gene, transportation and processing of the transcript, translation of the mRNA and post-translational modification of the translation product. Several regulatory mechanisms contribute to the reliable expression of an endogenous gene, regulating its temporal, spatial or inducible expression. Regulation of gene expression at the RNA level includes transcriptional control as well as post-transcriptional modification. Initiation of RNA synthesis is based on the recognition of *cis* acting sequences located within the region 5′ to the coding region (Maniatis et al., 1987). As binding of transcription factors to *cis* regulatory sequences regulates the activation of RNA transcription, the presence of particular binding proteins and the accessibility of *cis* regulatory sequences for binding proteins are two factors that determine the efficiency of transcription initiation. Both the chromatin structure (Felsenfeld, 1992) and the methylation state (Doerfler, 1983) of a promoter region can influence this efficiency in eukaryotic systems. While in *Drosophila*, gene inactivation corresponds to a condensation of chromatin (Shaffer et al., 1993), DNA-methylation plays an important role in the regulation of mammalian and plant genes, probably also via the assembly of methylated DNA into an inactive chromatin structure (Keshet et al., 1986). In plants, up to 30% of cytosines are methylated at carbon 5 (Adams & Burdon, 1985). Methylated C residues are located within CpG or CpNpG sequences, respectively (Gruenbaum et al., 1981). It has been proposed that methylation of symmetrical sequences provides a signal for a maintenance methylase that methylates C residues in a newly-synthesized strand located within symmetrical sequences, if the opposite strand carries a $m^5C$ residue in the complementary sequence (Holliday & Pugh, 1975). An inverse correlation between gene transcription and cytosine methylation has been observed for several plant genes. The tissue-specific transcription of maize storage protein genes (Bianchi & Viotti, 1988) and C4 photosynthesis genes (Ngernprasirtsiri et al., 1989) correlates with hypomethylation of the genes in tissue where they are transcribed. Differences in DNA-methylation do not necessarily occur within a gene or its promoter region. For the cell-specific transcription of the PEPCase gene of C4 plants, transcription corresponds to demethylation of a region located 3.3 kb upstream of the gene (Langdale et al., 1991). A connection between DNA-methylation and changes in chromatin structure in plants is documented for rRNA genes in wheat (Thompson & Flavell, 1988), where rRNA genes at different *nor* loci are expressed at different levels. Nuclear dominance of particular *nor* loci is associated with both undermethylation and an increased sensitivity to DNase I digestion.

Although it is still a matter of debate whether DNA-methylation is the cause or the consequence of inactivation of transcription, at least in mammals the importance of DNA-methylation in embryonic development has been convincingly shown. A mutation of the murine DNA methyltransferase that led to a 3-fold reduction in levels of genomic $m^5C$ levels, caused abnormal development and lethality in embryos (Li et al., 1992).

There is growing evidence that post-transcriptional mechanisms, which dominate the control of chloroplast gene expression (Berry et al., 1985), also play an important role in the regulation of nuclear gene expression (Gallie, 1993). On the RNA level this may involve pre-mRNA processing and transcript stability. Certain DNA elements, located downstream from the transcriptional start site, probably affect gene expression by regulating RNA stability (Green, 1993). It has been shown that different 3′ end regions strongly influence the level of gene expression, influencing the efficiency of 3′-processing or mRNA stability (Ingelbrecht et al., 1989). The DST element, a highly-conserved sequence located in the 3′ untranslated regions of a group of unstable soybean transcripts, has been identified as the cause for transcript destabilization (Newman et al., 1993). DST elements inserted into the 3′UTR region

of reporter genes induced the rapid decay of reporter transcripts.

## Mechanisms of transgene inactivation

Considering the fact that gene expression is regulated by a multilevel control mechanism, it follows that this regulation also affects the expression of recombinant genes introduced into the plant genome. Several reports confirm inactivation of transgene expression in which either initiation of transcription or transcript stability is involved.

Early studies on the activity of T-DNA genes in crown gall tumour lines, revealed a correlation between DNA-methylation and inactivity of T-DNA genes in certain lines (Amasino et al., 1984). Apparently T-DNA that integrates randomly into the genome became methylated at certain integration sites. The fact that certain copies of a transgene became hypermethylated while others remained hypomethylated and transcriptionally-active, strongly suggests that the degree of DNA-methylation is determined by the integration region. This position-specific inactivation could revert either spontaneously or after treatment of the cell line with the demethylating agent 5-azacytidine (Van Slogteren et al., 1989).

Another inactivation phenomenon was discovered when a transgenic tobacco line was retransformed with a construct that carried partial homologies to the construct already present in the genome (Matzke et al., 1989). In 15% of the double transformants, unidirectional inactivation of the first transgene occurred. This *trans*-inactivation was accompanied by hypermethylation of the promoters of the inactivated genes and was reversed when the two interacting loci were segregated from each other in progeny plants. Reversion, however, did not occur immediately, but gradually during the development of progeny plants. Occasionally partial methylation could even persist for two generations (Matzke & Matzke, 1991). Apparently inactivation in *trans* was dependent on homology between the two ectopic transgenes as well as the position where the transgenes were integrated. Inactivation not only occurred after a second transformation step, but it was also observed when two transgenic lines harbouring partial homology were crossed. Methylation of a target locus was again dependent on the integration region of the modifier locus and was more efficient when the target locus was homozygous (Matzke et al., 1993). Several mechanisms have been proposed to account for homology-dependent trans-inactivation. It was suggested that trans-inactivation reflects nonreciprocal competition for a transcriptional activator that was not freely diffusable (Matzke & Matzke, 1990), that it is due to transient ectopic pairing between the homologous transgenes accompanied by an exchange of chromatin components (Jorgensen, 1991) or that it derives from the interaction of anti-sense transcripts generated by transcription of antisense molecules from endogenous promoters at the 3′ end of the integration region (Grierson et al., 1991).

In contrast to the nonreciprocal interaction between ectopic transgenes, several co-suppression phenomena have been described that include the mutual inactivation of two partly homologous transgenes (Jorgensen, 1990). DNA-methylation may be involved in certain co-suppression effects (Linn et al., 1990), while for others no correlation between hypermethylation and gene inactivation was observed (Goring et al., 1991). Co-suppression is not restricted to transgenes, but can also affect endogenous genes. When the petunia chalcone synthase gene (*chs*) or the dihydroflavonol reductase gene (*dfr*) were introduced into *Petunia hybrida*, both the transgene and the endogenous gene were suppressed (Napoli et al., 1990; Van der Krol et al., 1990). Other examples include co-suppression of a tomato polygalacturonase gene (Smith et al., 1990) and tobacco chitinase (Hart et al., 1992) and glucanase (De Carvalho et al., 1992) genes. At least most co-suppression phenomena described so far seem to be due to post-transcriptional regulation requiring transcription of the transgene. It has been suggested that expression above certain threshold levels induces a biochemical switch into a new stable state of lower gene expression. This biochemical switch hypothesis, first described in relation to hormone habituation (Meins, 1989), provides an attractive explanation especially for co-suppression phenomena that are strictly correlated with homozygosity and where steady state levels of RNA, rather than transcription, are affected (Hart et al., 1992; De Carvalho et al., 1992). Although not all co-suppression phenomena can be explained by one mechanism, it is most likely that post-transcriptional degradation is involved in some of the effects described.

## Parameters influencing A1-expression in *Petunia hybrida*

The different inactivation phenomena that were observed for transgenic petunia plants carrying the

maize A1-gene seem to be exclusively due to inactivation of transcription that was accompanied by hypermethylation within the promoter region. So far we have not detected any evidence for post-transcriptional modifications. After direct DNA-transfer of an A1 gene linked to the viral CaMV 35S promoter into petunia protoplasts, primary transformants were regenerated that could be classified into three groups: plants with whitish flowers without any indication of A1-activity, transformants with salmon-red flowers exhibiting continuous A1-activity and plants carrying variegated flowers with A1-activity present in some floral cells. The uniformity of floral pigmentation was inversely correlated with the number of integrated A1-copies, as integration of multiple A1-copies predominantly created transformants with a whitish or variegated floral phenotype. Methylation analysis, using methylation-sensitive restriction enzymes, detected hypermethylation within the promoter region of multiple-copy transformants. The promoter of single copy plants, especially a *HpaII*-restriction site at the boundary of the promoter and the A1-cDNA, remained unmethylated (Lam et al., 1989). This *HpaII*-site is located close to a 21-bp element containing two TGACG motifs that bind the activation sequence factor (ASF-1) required for the maximal expression of the promoter (Lam et al., 1989). Although the measurement of DNA-methylation is limited to a few restriction sites, the *HpaII* site next to the ASF-1 binding sequence could be used as an indicator for promoter-specific methylation that inhibited A1-transcription.

To avoid co-suppression by multiple A1-copies, we further concentrated on the analysis of single copy transformants. Although the integration of one transgene improves the chance for universal expression, it is not sufficient. Three transformants (lines 16, 17 and 24) were selected, each carrying one complete transgene copy integrated at different chromosomal loci. In the white line 16 no A1-transcription was detectable, lines 17 and 24 showed A1-transcription at high and low intensity, respectively. In line 16 the inactivated transgene had integrated into a highly-repetitive genomic region. The weakly-expressed transgene in line 24 had integrated into a unique region that was highly methylated. The most intensively-transcribed transgene in line 17 was inserted into a unique region that was hypomethylated. The characteristic hyper and hypomethylation patterns of the integration regions in lines 24 and 17 were also imposed on the border region of the transgenes (Pröls & Meyer, 1992). Apparently the methylation state of the integration region is, at least in some part, responsible for position-dependent differences in gene expression.

To find out if integration of a single transgene into a hypomethylated genomic region was sufficient to guarantee stable expression, line 17 was further analysed. About 30,000 isogenic F1 plants derived from continuous pollination of homozygous line 17 were grown in the field. While blossoms on plants flowering early in the season were predominantly red, up to two-thirds of the later flowers on the same plants showed a reduction in A1-expression. Again, the reduction in A1-activity correlated with an increase in methylation. More than 95% of the greenhouse population of line 17, but only 37% of the field population, showed stable activity of the A1-gene, apparently due to environmental effects in the field. Interestingly, an endogenous factor seemed to determine the susceptibility of plants to this environmental stimulus. Progeny derived from early pollination of young parental plants was almost insensitive to inactivation, while in progeny from older flowers developed on the same parental plants, A1-expression was considerably reduced (Meyer et al., 1992). If an increasing degree of DNA-methylation is imposed on the transgene with increasing age of the parental plant, such imprinted methylation patterns would be transferred to the progeny. Progeny from older flowers would be more receptive to environmental stimuli as they carry a higher methylation density already.

For practical breeding, this model implies that breeders should use very young material for pollination to avoid selection of transgenes that might already have developed hypermethylation patterns. Transgene inactivation after exposure to environmental stimuli has also been observed in other systems, where tissue culture (Renckens et al., 1992) and heat stress (Walter et al., 1992) functioned as the environmental stimuli.

A detailed analysis of the transgene and its chromosomal integration region in several derivatives of line 17 implied that hypermethylation is limited to the transgene DNA only, while the hypomethylation state of the integration region remains unaltered (Meyer & Heidmann, 1994). The data indicate the likelihood of a DNA-methylation mechanism, which specifically recognizes foreign DNA. Such a mechanism, which has already been proposed for animal systems (Bestor, 1990; Doerfler, 1991), might recognize foreign DNA by its composition. It has been shown that the nuclear genomes of angiosperms are mosaics of long, compositionally homogeneous DNA segments, called isochores (Salinas et al., 1988) and that isochores contain defined GC contents of functional genes and their

chromosomal environment (Matassi et al., 1989). The A1 transgene in line 17 differs significantly in its AT content from the chromosomal environment. The integrated plasmid DNA has an average AT content of 47.5%, whereas the neighbouring 269 bp at the 5′ end and 196 bp at the 3′ end show an average AT content of 74% and 77%, respectively. It may therefore be conceivable that the isochore composition of a transgene has to match its integration regions to avoid specific methylation of the transgene.

The hypermethylated transgene not only inhibited its own A1-transcription, but also exerted a paramutagenic influence on a homologous allele (Meyer et al., 1993). The term 'paramutation' has been coined to describe a heritable change in gene function directed by an allele, more specifically, the inactivation of a paramutable allele by a paramutagenic allelic homologue (Brink, 1956). When a hypermethylated allele and a hypomethylated allele were combined by crossing, the hypomethylated allele becomes methylated and silenced in a semi-dominant way. The paramutation-like behaviour is possibly due to an interaction between the two differentially methylated alleles. Such interaction could be mediated by transient pairing of the two alleles. Because we also observe a higher chromatin condensation in the hypermethylated allele (Meyer et al., 1994), we suggest that an exchange of methylation patterns also includes a change in chromosomal components. Assuming that transient pairing might not only occur between allelic homologues, but also among certain ectopic transgenes some trans-inactivation phenomena could include transient somatic pairing and an exchange of chromatin components. An improved understanding of the chromatin structure of plant genes is required to anticipate its importance in gene expression.

## Approaches towards stabilization of transgene expression

Although the mechanisms involved in the regulation of gene expression are only partially understood, it is justifiable to say that multiple factors have to be controlled to increase the chance of stable expression of a transgene. These parameters include the number and structure of integrated copies, the chromosomal integration region, its chromatin structure and methylation state and the strength and specificity of promoter elements. Also included in such a parameter list should be DNA elements within the transgene that might function as recognition sequences for DNA-methylation or increased mRNA-degradation. This list, although incomplete, should serve as a guide for the selection of appropriate transgenic plants.

A desirable characteristic for any transgenic plant is single copy transgene integration. Most transgenic plants existing today have been generated using *Agrobacterium* strains that transfer their T-DNA to plant cells where it is randomly integrated into the genome (Zambryski et al., 1983). Because the T-strand is protected by bacterial proteins that also may facilitate its passage into the nucleus, T-DNA is frequently integrated without large internal deletions. Due to the mechanism of illegitimate integration (Mayerhofer et al., 1991), however, T-DNA insertions are often rearranged, preferentially in the junction region between T-DNA and chromosomal DNA (Simpson et al., 1982). Only one third of T-DNA transformants carry insertions at one locus (Koncz et al., 1989). Among single-locus insertions up to 60% of the plants may contain dimeric or trimeric inserts (Jorgensen et al., 1987; Jones et al., 1987). On average only one in ten transformants will contain a single integrated T-DNA copy. To avoid unintended co-suppression effects, such simple integration events should be selected and examined in more detail for major rearrangements and short target site duplications.

Direct DNA transfer into protoplasts and microparticle bombardment frequently lead to random integration of truncated copies of plasmid-DNA. The integration pattern seems to be mainly influenced by the structure of the transferred DNA. More than 50% of tobacco transformants derived from PEG-mediated transfer of supercoiled plasmids contain a single, although still truncated copy of the transgene. Early research on direct gene transfer (Davey et al., 1989) has mainly focused on the enhancement of transformation frequencies without paying much attention to the complexity of integration patterns and the long-term stability of transferred genes. Considering the possible expression-instability generated by multiple copies, any method aiming at an enhancement of transformation frequencies should also be evaluated for a conservation of a high proportion of single copy integration events, otherwise many transformants have to be scanned for a single integration event.

As mentioned previously, the integration region has an important influence on the methylation pattern and expression of the integrated transgene. The methylation state of the integration region or of its repetitiveness should therefore be examined. T-DNA preferen-

tially integrates into chromosomal loci that are potentially transcribed, which reduces the probability for a negative influence of the integration region. Recently the stable expression of a *rolA* transgene in *Arabidopsis thaliana* was confirmed, when 425,000 individuals were tested (Dehio & Schell, 1993). Confirmation of stable *rolA* expression, which monitors somatic instability very precisely, demonstrates that at least certain transgenes that are free from inactivating influences of the integration region can be selected. However, the plants were not tested under varying environmental conditions.

In considering the specific methylation of a transgene DNA without detectable changes in the methylation pattern of the chromosomal environment (Meyer & Heidmann, 1994), we cannot rule out the fact that some recombinant constructs contain specific features that allow its recognition as foreign DNA by plant enzymes. It will be interesting to clarify this phenomenon and to define which characteristics of a transgene and an integration region contribute to stable expression. If the very low efficiencies for gene targeting into the plant genome (Paszkowski et al., 1988) can be improved, this would allow the selection of suitable integration regions into which transgenes might be targeted. As an alternative, recombinant genes could be transferred together with suitable chromosomal flanking regions. Scaffold attachment regions (SARs) which have been positioned 5′ and 3′ to a marker gene have stabilized transgene expression in animals (Stief et al., 1989) and plants (Breyne et al., 1992). The formation of chromosomal loops by SARs that connect genomic DNA to the nuclear matrix might isolate the transgene from the influences of the integration region. Another approach to uncouple transgene DNA from negative position effects could be its positioning on extrachromosomally replicating vectors (Meyer et al., 1992), although this will probably impose restrictions on the size of foreign DNA that can be propagated in transgenic plants.

## Outlook

To adopt transgenic plants for agriculture, the participation of plant breeders seems essential. Transgene inactivation based on changes in chromatin structure are probably dependent on the genetic background. It can be expected that many different genes encoding chromatin proteins determine the degree of condensation. In *Drosophila* about 200 loci have been mapped that act either as suppressors or enhancers of position effect variegation. Transgenes repressed in one line might be active in another genetic environment and stabilization might be established in a classical breeding and selection process. As mentioned above, transgene inactivation might also occur by post-transcriptional degradation that is initiated at certain threshold levels. To avoid post-transcriptional degradation, lines would have to be selected that transcribe a transgene at moderate, but constant levels. Finally, extensive field studies will be required to assess the influence of various environmental conditions. Although molecular breeding does simplify the introduction of new traits into plants, the necessity for stable activity of the transgene requires a final evaluation of transgenic lines by the breeder.

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